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Automatic Measurements of α-Amylase Activities during γ-Irradiation

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A gamma-ray irradiation facility equipped with an automatic analyzer, a liquid flow circulating system, and a regulating system of irradiation temperature, has been installed in Kyoto University. This instrument enable to analyze chemical changes and biological effects by irradiation automatically during irradiation without any interrupting of the irradiation throughout experiments. Instrumentation and application to measure activities of an enzyme before, during and after γ-irradiation are described.

INTRODUCTION

For biochemical and chemical research a γ-ray irradiation facility has been installed in Kyoto University[1,2] and used for several chemical and enzymatic studies by the authors.[3-16] This irradiator has been made several arrangements which enable to supply moderate and continuous irradiation to chemical and biochemical systems, to regulate irradiation temperature, and to deliver flowing liquid materials by a circulating system. Further arrangements for the equippment of automatic chemical analysis were made by combining an Autoanalyzer and a circulating pump with the facility. It has been possible to analyze chemical reactions and actual absorbed dosages in the media, to make self-recordings of chemical changes or biological phenomena in the circulating system, and to measure enzyme activities without any interrupting of the irradiation throughout the experiments.

The automatically measured activities have been studied comparing with the studies in which the activities of the enzyme were determined before and after exposing to γ-irradiation statistically in another facility of the Institute of Chemical Research, Kyoto University[17]. This paper deals with the arrangements of the automatic measurements and an application to studies on the inactivating mechanism of α-amylase by γ-irradiation.

INSTRUMENTATION

Gamma-ray Irradiation Facilities

A γ-ray irradiation facility[1]*** containing cobalt-60, 100 curies, designed by

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*** Toshiba RE1010 Irradiator is now available in commercial from the Toshiba Ltd., Tokyo.

the authors and installed at Kyoto University, was used. This irradiator is equiped with a temperature control system in an irradiation chamber, which enable to maintain irradiation temperature at a range of 5 to 95°C, and with a circulating system for flowing liquid materials during irradiation as shown in Photo. 1, 2 and 3. The radiation source, ten pellets of cobalt-60 encapsulated in a double cylinder (5.5 mm in diam. and 75 mm in length), moves vertically down from the mounted container to 1.3 cm below from the bottom of the cylindrical irradiation chamber (30 cm in diam. and 20 cm in height). In the irradiation chamber of this irradiator, surrounding spiral tubes in which flowing liquid may becirc ulating for irradiation, are at 12 cm from the center of the chamber in this experiment.

The dosimetry in the chamber has been reported separately[1] and the average dose rate is 4.27 kR. per hour in the circulating pipes. This dose rate is variable by changing the source position from the bottom of the chamber, and a vertical distribution in the chamber is from 3.84 to 4.70 kR. per hour at 12 cm from the center of the chamber, in which the source is at the bottom. Another γ-ray irradiation facility[17] containing cobalt-60, 2 kilocuries, installed in the Institute of Chemical Research, Kyoto University, was used for reference experiments.

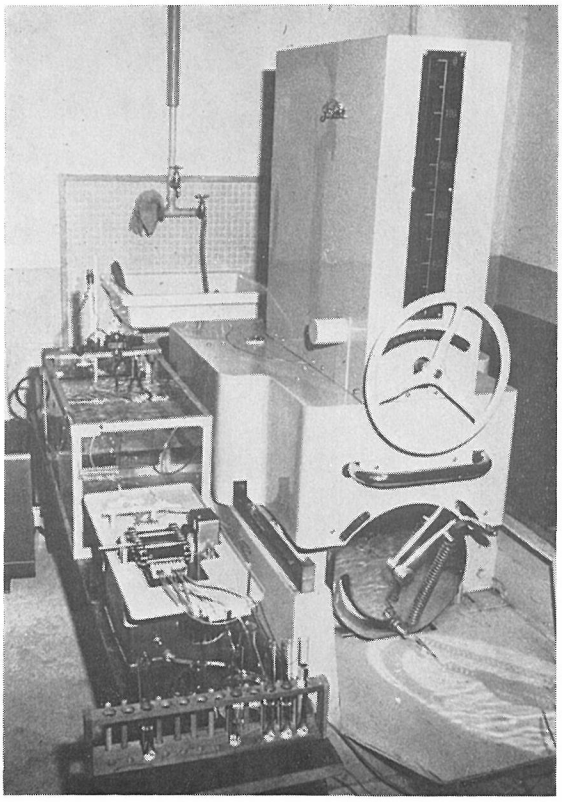

Photo. 1. The γ-ray irradiation facility equipped with an automatic analyzer.
Toshiba model RE1010 γ-ray irradiator and a Technicon Autoanalyzer.

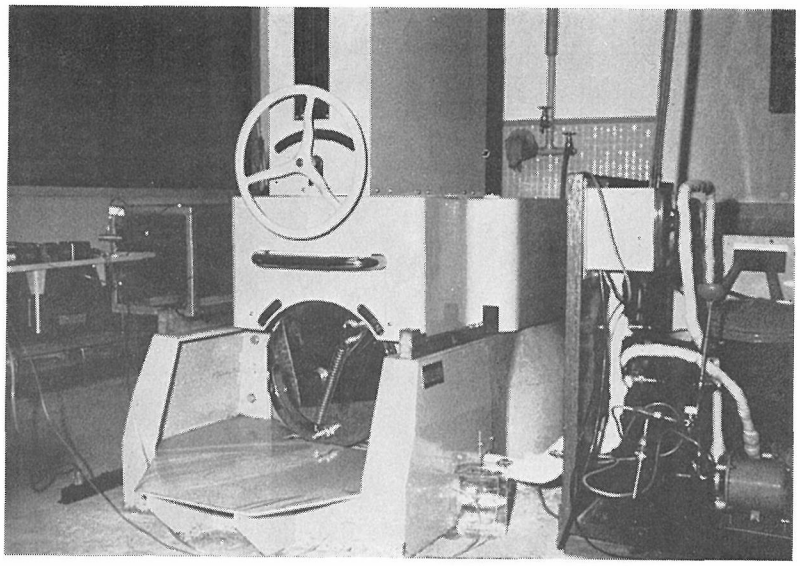

Photo. 2. The γ-ray irradiation facility equipped with a circulating system of samples and reagents, and a regulating system of temperature during γ-irradiation.

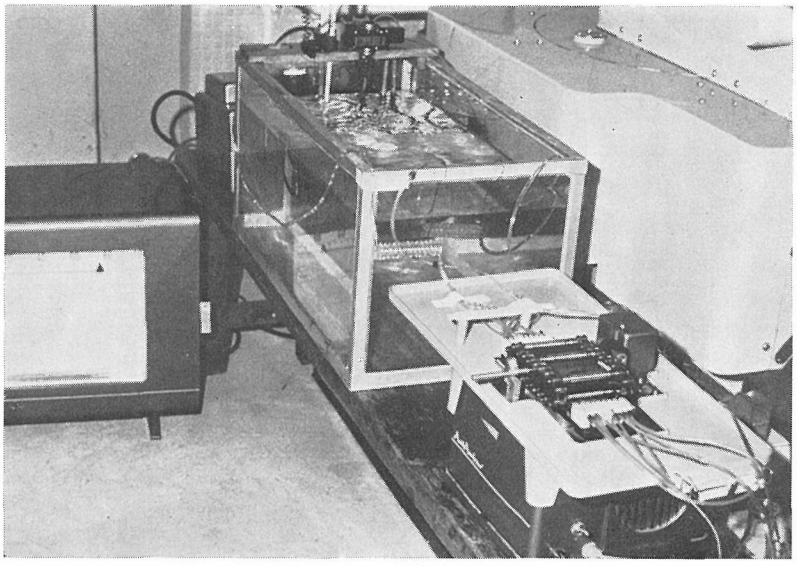

Photo. 3. The automatic analyzer consisted of a reagent delivery, a peristaltic pump, a reaction bath and a recorder, with which equipped the γ-ray irradiator.

Circulating System for Continuous Irradiating Flow Samples

Circulating system for continuous irradiation is shown in Fig. 1. A delivery pump, which is usually used in an automatic liquid chromatograph of Japan Electron Optics Co., Tokyo, was used in this experiment. This pump consists of glass cylinder which appeared to be safe for the determination of enzyme activities

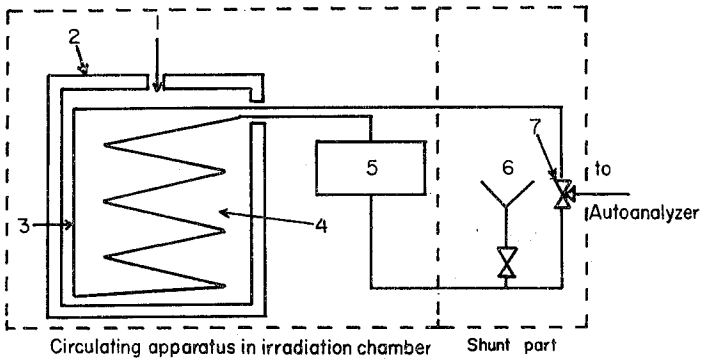

Fig. 1. Shematic diagram of the circulating system of sample and reagents in the γ-ray irradiator, with which equipped an automatic analyzer. 1: Inlet of Cobalt-60 radiation source; 2: Cover of the facility; 3: Tubing for circulation of sample and reagents; 4: Irradiation chamber; 5: Circulation pump; 6: Inlet of instiling sample; 7: Three-way cock and outlet of sample to Autoanalyzer.

and can deliver any liquid at a flow rate from 4.53 to 0.19 ml per min. For all piping polyvinyl tubes were used in order to avoid the inactivation of the enzyme which has been examined to be sensitive to ferric ion dissolved out from stainless steel pipes even at the concentration of 10^{-3} M. In the irradiation chamber, a polyvinylpipe, 2 mm in diam. and 5.6 m in length, can deliver a sample solution from the inlet to the outlet of the circulating pipe at a flow rate of 4.5 ml per min. by the pump. It takes 25 minutes for the irradiation of one cycle of the circulation at a flow rate of 4.5 ml per min. in the pipe.

Automatic Analyzer for Chemical Measurements

For automatic analysis and self-recording of the results of chemical measurements during irradiation, an Autoanalyzer of Technicon Corp., Chancy, N.Y. was used in this system as shown in Photo. 2. The Autoanalyzer consists of a propor-

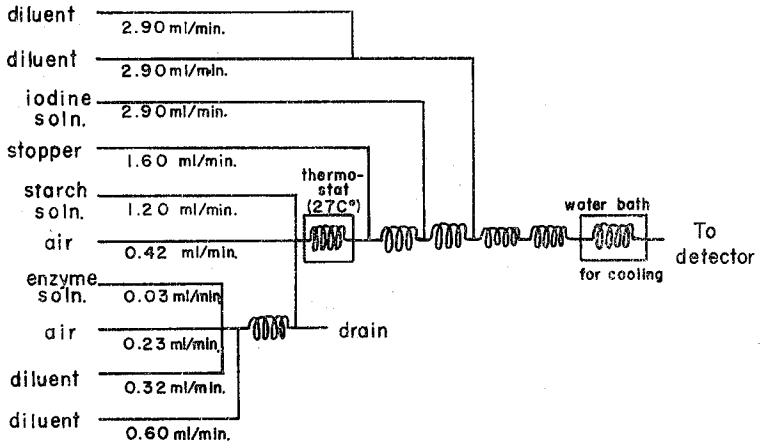

Fig. 2. Schematic diagram of automatic measurement of activity of α-amaylase during γ-irradiation.

tional pump, a photometer with a flow cell of 1.5 cm light path and a 570 nm filter, a single pen recorder, a thermostated bath for reactions, a plastic cooling bath, vinyl pipes, glass cactuses, and glass mixing coils. A typical system of this automatic measurements is shown in Fig. 2.

EXPERIMENTAL

Materials

Crystalline specimen of bacterial liquefying α-amylase of *Batilus subtilis* was obtained from Daiwa-kasei Co., Osaka. Chromatographic purification of the preparation was carried out by gel filtration on a Sephadex G–50, 0.9×50 cm column excluding excess calcium acetate and sulfate and by checking the purity using electrophoresis with a cellulose membrane.

The purified preparation from which a stabilizer, calcium acetate, was excluded, was easily denatured during circulation in the equipment even without irradiation. The denaturation of the enzyme in the solution, 5 μg per ml in concentration, was examined preliminarily to be 51.5 per cent inactivation for 1 hour and 66.2 per cent for 2 hours. In order to avoid the denaturation 1/500 molar calcium acetate per 1 molar protein was added to the preparation by gel filtration using a 1/500 M calcium acetate solution. Denaturation of the preparations in calcium acetate, 2 to 5 μg per ml in concentration, was found to be within 1 per cent for 1 hour in this circulating system.

Soluble starch as a substrate of α-amylase, iodine for colorimetric determination of the starch, and all other reagents were purchased from Nakarai Chemicals Co., Kyoto.

Methods

Dosimetry in the Circulating of Flowing Liquid: Fircke's ferrous-ferric chemical dosimetric solutions circulated into the irradiation chamber were used for the dosimetry during the circulation of the solutions. Optical absorption of ferric ions produced in the irradiated solutions was determined at 512 nm colorimetrically by an Autoanalyzer equipped with the irradiation facility. The absorbed dosages measured automatically were within 95 % to 105 % of those determined by a usual chemical dosimeter.

Continuous Irradiation of the Preparations of α-Amylase and Measurements of Activities of the Enzyme: A continuous irradiating system consists of a γ-irradiator, a circulating system and an Autoanalyzer. Typical recordings of the activities of α-amylase during γ-irradiation are presented in Fig. 3. Preparations of α-amylase were 100 μg per ml in concentration for the continuous irradiation, and 3 μg per ml for determination of the activities. Concentration of the substrate, 120–150 μg of starch per ml, depended upon that of the enzyme, 1 to 100 μg per ml of the enzyme solution. For stopping the enzyme reaction an 0.3 N acetic acid solution was added, and for color developping an iodine solution, 200 μg per ml, was used at 27°C and pH 5 to 6 for 2 min. and 40 sec. Final concen-

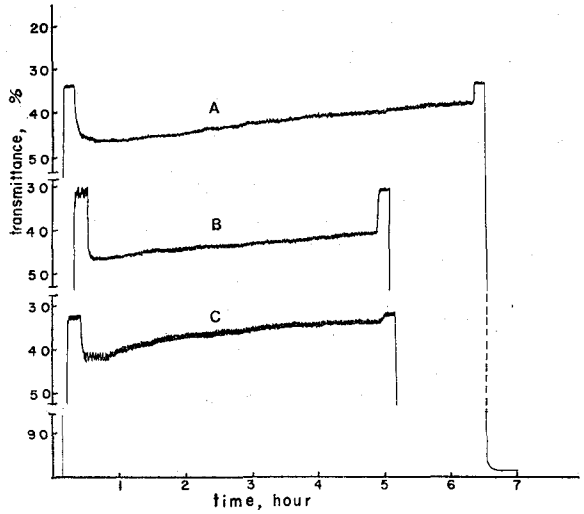

Fig. 3. Automatic recording of activities of α-amylase during γ-irradiation. A: in 1/500 M calcium acetate solution; B: in 1/10 M calcium acetate solution; C: in 1 M calcium acetate solution.

tration of the enzyme solutions was in a region of 0.6 to 3 μg per ml, keeping a linear relationship of the enzyme activities to the substrate concentration of 280 to 600 μg per ml. The amount of starch and the recorded iodine color yield have kept a quantitative relationship throughout this determination, as checked by a preliminary test. An iodine solution of 100 μg per ml and a starch solution of 20 to 100 μg per ml were mixed and transmittance of the reaction mixture was recorded automatically. Absorbance was calculated from the recorded transmittance for the quantitative determination of residual starch concentration depending on the enzyme activity. Quantitative results were obtained by a way of measurements of peak height of the recorded signal. Measurements of peak area on half-height's width were also found to give similar quantitative results checked preliminarily.

RESULTS

1. Dosimetry in the circulating system

In the irradiation chamber dosimetric distribution was determined by a usual chemical dosimeter, and in the circulating pipes running for the irradiation, was by

Table I. Dose Rates in the Circulating System*.

Irradiation time in min.	92	184	368	736
Dose rate $\times 10^{-3}$ in rad	4.01	4.30	4.30	4.41
Average dose rate in rad		4.27×10^3		

* For one cycle of the sample solution in the circulating system in which the circulation pipe is 12 cm distance from the radioactive source, it takes 25 minutes of which 23 minutes for the γ-irradiation. Mean dosages in the chamber are 5.53×10^4, 1.72×10^4, and 5.12×10^3 rads per hour at 3, 6, and 12 cm distance from the source respectively.

automatic measurements of ferrous ions in the dosimetric solutions. The results of the circulating dosimetry are presented in Table I.

2. Measurements of Enzyme Actitvities of the Flowing Preparation Irradiated in the Circulating System

Activities of the enzyme solutions in the circulating system were measured before and during irradiation. Natural inactivation during the circulation with and without a calcium stabilyzer is presented in Table II.

Table II. Inactivation of α-amylase during the Circulation without Irradiation in the Presence or Absence of a Calcium Stabilyzer

Condition	Circulation time hr	Inactivation %
In a glass cylinder pump[a)]	1[f)]	0.5
and in a vinyl pipe[c)]	1[g)]	51.5
	2[g)]	66.2
	1	6.8
	2	8.1
	3	11.2
In a glass cylinder pump[a)]	1	14.6
and in a stainless steel pipe[d)]	1.5	14.6
In a stainless steel rotary pump[b)]	1	66.4
and in a vinyl pipe[e)]	2	75.1

At a flow rate of a); 4.53, b); 3.50, ml per min. after washing with an 0.1% dimethylamine solution. In diam. and in length of c); 2 mm×5.6 m, d); 4 mm×3.6 m, and e); 6 mm×0.5 m. All are with an 0.2 mM except to f); and g); f); with a 2 mM calcium acetate solution and g); with none.

For determination of the enzyme activities, the flowing system was used as shown in Fig. 2. α-Amylase was dissolved into 0.1 M calcium acetate and 0.1 M sodium chloride stabilizer in concentration of 2.448×10^{-2} to 0.490×10^{-2} mg per ml. Soluble starch was dissolved into 2.302×10^{-4} M acetic acid and 3.684×10^{-3} M sodium acetate buffer in concentration of 97.85 μg per ml. The enzyme solution mixed with two diluents at flow rates of 0.32 and 0.60 ml per min., was run at a flow rate of 0.03 ml per min. and mixed with air at a flow rate of 0.23 ml per min. The resulted enzyme solution was mixed together with the starch solution which was at a flow rate of 1.20 ml per min. and was mixed with air at a flow rate of 0.42 ml per min., in the thermostated mixing coil at 27°C. The enzyme reactions in the resulted mixture were stopped by mixing with the 0.3 N acetic acid solution at a flow rate of 1.60 ml per min. To the enzyme solution, an iodine solution of 200 μg per ml was added for color developping of residual starch. After mixing and color developping, two diluents at a flow rate of 2.90 ml per min. were added to the resulted mixture and allowed to further mixing and standing for cooling in a water bath. Measurements of the developed color were carried out by a Technicon photometer with a filter of 570 nm. Natural inactivation during the

circulation was observed for 5 hours on preparations of the enzyme concentration of which was 1, 3, and 20 μg per ml in the 1/500 M calcium acetate solution. As showing in Table II, the natural inactivation was found to be within about 10 %.

Stabilizing effects of calcium ion on α-amylase were examined on 1/500, 1/100, 3/10, 1/10, 1/5, 1/2 and 1 M calcium acetate solutions of the enzyme. Typical three inativation curves were obtained from these measurements as shown in Fig. 4 and 5, within 5 % errors. More concentrated, 1 M, 1/2 M, and 1/5 M calcium acetate solutions showed reverse-S-shape, medium 1/10 M showed linear, and dilute 3/100, 1/100, 1/500 M showed hyperbola curves, as shown in Fig. 5. These results showed that stabilyzing effects of calcium acetate on α-amylase played an important role in the inactivation of α-amylase in solution by γ-irradiation.

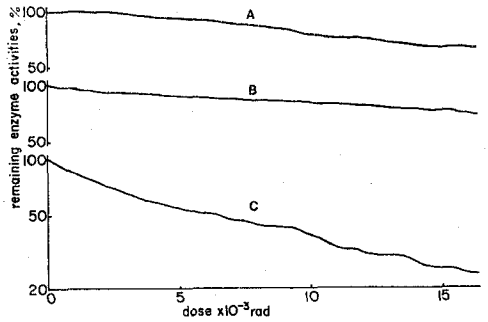

Fig. 4. Inactivation curves of γ-irradiated α-amylase, measured A: 1 M; B: 1/10 M; C: 1/500 M, calcium acetate solution with the automatic analyzer during γ-irradiation.

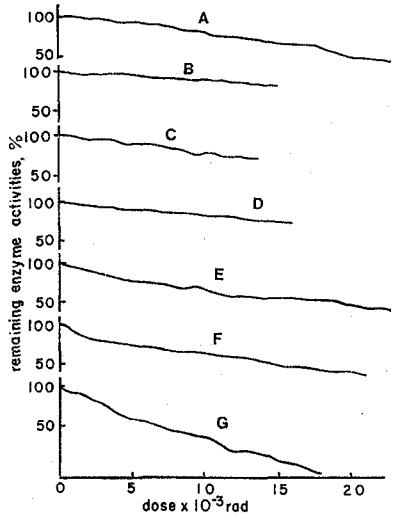

Fig. 5. Inactivation curves of γ-irrirradiated α-amylase, measured with the automatic analyzer during γ-irradiation.
A: 1 M; B: 1/2 M; C: 1/5 M; D: 1/10 M; E: 3/100 M; F: 1/100 M; G: 1/500 M, calcium acetate solutions.

Effects of concentration of α-amylase on the irradiation in solution of 1/500 M calcium acetate were examined as shown in Fig. 6. All inactivation curves showed hyperbola but less inactivation was found on the concentrated solution of the enzyme. Inactivated molecules were calculated at a dose of 10^4 rads according to a following equation:

$$\text{Inactivated molecule, mole/ml} = \frac{\text{conc. g/ml} \times \text{inactivation } \% \times 6.023 \times 10^{23}}{\text{Molecular weight of } \alpha\text{-amylase, } (=47{,}500)}$$

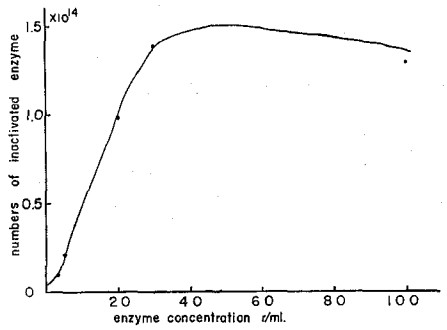

Fig. 6. Concentration effect of α-amylase to γ-ray inactivation.

As given in Fig. 6, plotting numbers of the inactivated molecules vz. enzyme concentration of the irradiated solution showed a S-shape curve. Between 3 and 30 μg per ml of the concentration, the inactivated molecules increased with that of the concentration of the enzyme, but not below 3 or above 30 μg per ml comparatively. The results surely showed that the inactivation was caused indirectly in aqueous solution.

3. Effects of calcium chloride and of barium chloride on the γ-ray inactivation of α-amylase in aqueous solution

Effects of 0.1 M and 0.01 M calcium chloride and 0.5 M and 0.01 M barium

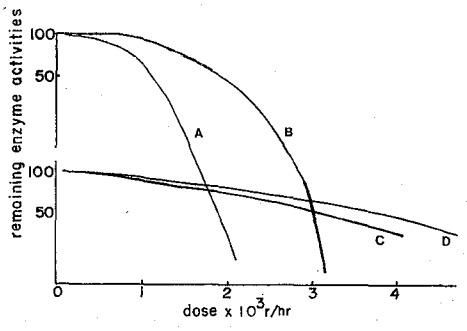

Fig. 7. Effects of calcium chloride and barium chloride to γ-ray inactivation of α-amylase.
A: 1/10 M calcium chloride; B: 1/2 M barium chloride; C: 1/100 M calcium chloride; D: 1/100 M barium chloride.

chloride on the inactivation of α-amylase in aqueous solution during γ-irradiation were observed as shown in Fig. 7. Radiosensitizing effects of the reagents were observed in the experiments.

DISCUSSION

Benefits of instruments of automatic measurements during irradiation without any interruption of the irradiation are that self-recording of chemical and biological changes throughout irradiation without dangerous exposure to ionizing radiation, detection of short lived intermediates of the changes during irradiation, troubleless performance of laborous measuring treatments of chemical analysis are possible together with accurate and reproduceable results. In this instrumentation it is not always easy to obtain the benefits successfully because of some limitation and unsuitability of the parts of the instrument and of procedures of the chemical measurements for enzyme reactions.

The gamma ray irradiation facility was only possible to irradiate in a range of dose 5.12×10^3 to 5.53×10^4 rads per hour. For larger dosages it is necessary to repeat the circulation of the liquid. This irradiation, however, is not always performed continuously under identical conditions throughout experiments because of taking out the irradiated materials at some intervals outside the irradiation chamber. Periods of the intervals were regulated by changing length of pipes. The pump of the circulating system can deliver only amounts of 0.19 to 4.53 ml per min. which limit circulating time necessary for irradiation and detection. It takes 25 minutes at least at the delivery rate of 4.53 ml per min. In order to detect intermediates of radiological process as quick as possible and to remove complicated effects of secondary irradiation products on the solutions, it is desirable to circulate the materials more rapidly on more delivery amounts of the materials. Piping should also be selected proper diameter and length for the experiments. Stainless steel pipes shoud not be used for the experiments because the enzyme are very sensitive to metallic ion, such as ferric ions which were dissolved out from stainless steel pipes.

The dividing system of irradiated materials, equipped with this instrument, had a compensation procedure by which air was introduced air caused to influence on the constant delivery and on the dosage of the circulation materials. Irradiation effects of oxygen in the air may be excluded by using nitrogen atmospheric conditions. To obtain the good results it was necessary to use the constant delivery pump which is useful for concentrated acidic or alkalline solutions.

Possibility of quantitative analysis was examined preliminarily on enzyme and substrate concentrations, and on experimental conditions of color developing. To obtain the sufficient results several arrangements were made in the measuring system, in which two diluents were added and mixed to the enzyme solution and to the iodine solution, and also seven mixing coil were connected with the running pipes for adequate dilution and complete the reaction. This instrumentation of course, was suitable for this enzyme reaction, and could be possible to measure any other enzyme and chemical reactions by changing the parts and their pipings.

Previous experiments have shown several mechanisms of irradiation of this enzyme such as a single or multiple hit mechanism under the various conditions. A most probable mechanism seems to be a single hit from the results of several kinetic studies on parameters such as Michaelis constants and maximum velocities before and after exposure to γ-irradiation. From these consideration it was proposed that a role of calcium acetate is a protective effect to attack by the OH radicals produced in the irradiated solution in addition of stabilyzing effects of calcium ion on the conformation of the enzyme molecule in solution. It may be that acetate ions react with the OH radicals to reduce inactivating effects of the OH radicals and to change to acetate radicals which appeared to be effective to the inactivation.

The protective effect of calcium acetate appeared on the hyperbola curve of inactivation, the scavenging effect of calcium acetate on the reverse-S shaped curves, and both resulted in the linear curves. Using this instrument further kinetic studies on radiation inactivation of α-amylase in the aqueous circulating system are now in progress for making clear the inactivation mechanisms of α-amylase by γ-irradiation and some application to studies of radiation induced chemical reactions are planning by the authors.

Extrapolating semilogarithmic inactivation curves of γ-irradiated α-amylase in the aqueous circulating system, 1.2 or 1.3 hit inactivation was obtained according to the hit theory. The hyperbola curves shown in Fig. 5, however, caused by a single hit effect together with effects of calcium acetate in the enzyme solutions.

Dilution effects were found in plotting numbers of molecules inactivated by exposure to 4×10^4 rad doses vz. concentration of the enzyme solutions. This effect showed an indirect mechanism of the enzyme inactivation. At lower concentration gradual inactivation may be caused by major recombination of effective radicals to the less amounts of enzyme molecules. At higher concentration similar gradual inactivation was observed owing to relatively less amounts of radicals to the much amounts of enzymes. Conclusively, indirect inactivation of α-amylase was observed according to a single hit mechanism. It looks like to be of importance and significant that the radiosensitizing effects of calcium chloride and barium choloride to γ-ray inactivation of α-amylase in aqueous solution were observed in Fig. 7. This should be reported in detail elsewhere.

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